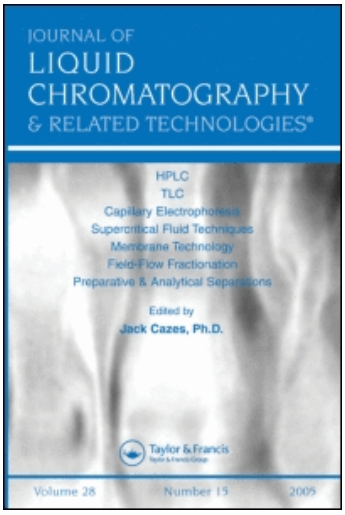

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EXPERIMENTALLY DESIGNED, VALIDATED HPLC SIMULTANEOUS DETERMINATION OF PRIDINOL AND DICLOFENAC IN THEIR COMBINED PHARMACEUTICAL FORMULATIONS, WHICH ALLOWS LIMITING DICLOFENAC RELATED COMPOUND A

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□ *The development and validation of an HPLC method for the determination of pridinol and diclofenac in their combined formulations and the simultaneous limit testing of diclofenac related compound A is described. The separation was performed on a C_{18} column. Experimental design and response surface strategies were employed for optimizing detection wavelength (225 nm) and mobile phase composition [MeOH:2-propanol:phosphate buffer (50 mM, pH 5.5), 48:9:43 (v/v/v), 1 mL min^{-1}], and for validation purposes. The method was successfully applied to the quality control of commercial brands of tablets and capsules. Found impurity levels were below 0.1% (LOQ= 0.02%). Stressed samples were also evaluated.*

Keywords diclofenac, diclofenac related compound A, experimental design, HPLC determination, pridinol, validation

INTRODUCTION

Diclofenac (DIC, Figure 1) is 2-[(2,6-dichlorophenyl)-amino-phenyl]-acetic acid, a synthetic non-steroidal anti-inflammatory agent, clinically prescribed for the treatment of inflammatory disorders, including rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis, and acute pain from sport injuries and other conditions.[1]

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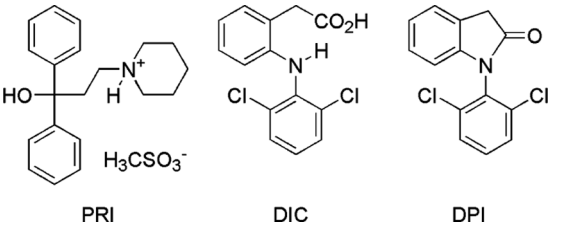

FIGURE 1 Chemical structures of pridinol mesylate (PRI), diclofenac (DIC), and diclofenac related compound A (DPI).

When exposed to heat,[2,3] light,[4] ultrasound,[5] or acidic conditions,[6,7] diclofenac may undergo an intramolecular dehydrative cyclization, leading to 1-(2,6-dichlorophenyl)indolin-2-one (DPI, Figure 1).[8] This is coded as Impurity A in the European Pharmacopoeia,[9] and as DIC related compound A in the USP.[10] DPI was also reported as a formulation impurity[11] and as a degradation product of the topical emulgel[12] and of DIC injectables subjected to terminal sterilization.[13]

The determination of DPI in DIC bulk drug and in pharmaceutical products containing DIC is a specific official requirement, contained in some of the leading Pharmacopoeias, which limit its presence.[9,10,14] Accordingly, the simultaneous quantification of DIC and DPI has been reported by densitometric means,[15] and the potential presence of the impurity was taken into account during the development of analytical methods for the determination of DIC in association with other pharmaceutically active compounds.[12,16]

On the other hand, Pridinol mesylate (PRI, 1-diphenyl-3-piperidinopropan-1-ol methanesulfonate) is a muscle relaxant.[17,18] This active ingredient (Figure 1) is usually marketed in association with nonsteroidal anti-inflammatory drugs, for treatment of muscular contractures and low back pain.[19] A stability-indicating assay of PRI[20] and the simultaneous quantification of PRI and meloxicam[21] have been recently reported.

The lack of a procedure for the quantification of pridinol and diclofenac in their pharmacological associations, and the official requirement to limit DPI as an impurity in all formulations containing DIC, prompted us to develop a HPLC method suitable for simultaneously accomplishing both of these purposes. Method optimization and validation stages were carried out employing experimental design techniques. This is a cost-effective and convenient approach to explore and optimize multivariate systems,[22] the use of which is encouraged by some official texts.[10]

EXPERIMENTAL

Chemicals

HPLC-grade solvents (J. T. Baker, Mexico), double-distilled water, and pharmaceutically-certified PRI and DIC (Droguería Saporiti, Buenos Aires, Argentina) were employed. All other reagents were of analytical grade (Merck, Darmstadt, Germany). Sodium phosphate solutions (50 mM) were prepared according to the USP 30.[10] The mobile phase was filtered through 0.45 μm nylon filters and degassed just before use. DPI was synthesized according to the literature[23] and spectroscopically characterized. Tablet and soft gelatin capsule products (50 mg DIC and 4 mg PRI) were purchased form a local drugstore. A granulate containing DIC and excipients was received as a gift from Mar Laboratories (Buenos Aires, Argentina).

Apparatus, Operating Conditions, and Software

Chromatographies were performed on a Varian Prostar 210 instrument consisting of two pumps, a manual injector fitted with a 20 μl loop and a Prostar 325 variable dual-wavelength UV-Vis detector. An HP 1100 liquid chromatograph equipped with a photodiode array detector was used for the specificity studies. The UV spectra of the drugs were acquired in a Shimadzu UV-1601PC spectrophotometer.

In the optimized method, the determinations were carried out on a Luna C_{18} analytical column (250 mm × 4.6 mm I.D., 5 μm particle size) thermostatted at 30°C. The mobile phase was a 48:9:43 (v/v/v) mixture of MeOH, 2-propanol and 50 mM sodium phosphate (pH = 5.5), pumped at 1.0 mL min^{-1}. Detection wavelength was 225 nm. All samples were filtered through 0.45 μm nylon filters before injection. The experimental designs, data analysis, and response surfaces were carried out in Design Expert v. 7.1 (Stat-Ease Inc., Minneapolis, MN). Statistical analyses were performed in SPSS v. 9 (SPSS, Inc., Chicago, IL).

Preparation of Standard Solutions

Stock standard solutions of DIC (1300 μg mL^{-1}), PRI (104 μg mL^{-1}), and DPI (4.0 μg mL^{-1}) were prepared in mobile phase. They were stored at 4°C in light-resistant containers and left to attain room temperature before use. Periodic HPLC analysis demonstrated their stability for at least 90 days.[24] Working solutions were freshly prepared in volumetric flasks, by mixing appropriate volumes of the corresponding stock solutions and

completing to the mark with mobile phase. The solutions were protected from light throughout the experiments.

Preparation of Samples for the Analysis of Tablets and Soft Gelatin Capsules

Tablets

Twenty tablets were weighed, crushed and mixed in a mortar. A portion of powder equivalent to the weight of one tablet was accurately weighed and dissolved in a 50 mL volumetric flask containing 25 mL MeOH. Sodium phosphate solution (pH = 5.5) was added to the mark. After mixing, a 10 mL aliquot was centrifuged (5 min at 1500 rpm), 3 mL of the clear supernatant were transferred to a 10 mL volumetric flask and made up to volume with mobile phase. The procedure was performed in triplicate for each brand.

Soft Gelatin Capsules

Twenty capsules were individually weighed and dissolved in 50 mL volumetric flasks containing sodium phosphate solution, pH = 5.5 (25 mL), completing to the mark with MeOH. 5 mL aliquots were taken from each flask and pooled; a 3 mL portion of the pool was transferred to a 10 mL volumetric flask, completing to the mark with the mobile phase. The procedure was performed in triplicate.

Preparation of Stressed Solid Samples

Twenty tablets were weighed and crushed in a mortar. Aliquots of the resulting powder were irradiated with a Philips HPA 400 lamp (24 h) and with a 200 Watt incandescent lamp (2 weeks), both placed at 20 cm from the samples. Other tablet powder aliquots and granulates containing DIC were submitted to dry heat (37°C and 80°C, 2 weeks) and also to 37°C and 85% RH (two weeks). For analysis, samples were prepared as described for Tablets.

RESULTS AND DISCUSSION

Method Development and Optimization

Detection Wavelength

Official procedures use single wavelength detection;[9,10,14] therefore, its selection was accomplished with the aid of spectral data of the analytes (Figure 2A) coupled to Derringer's desirability (*D*) function.[25,26]

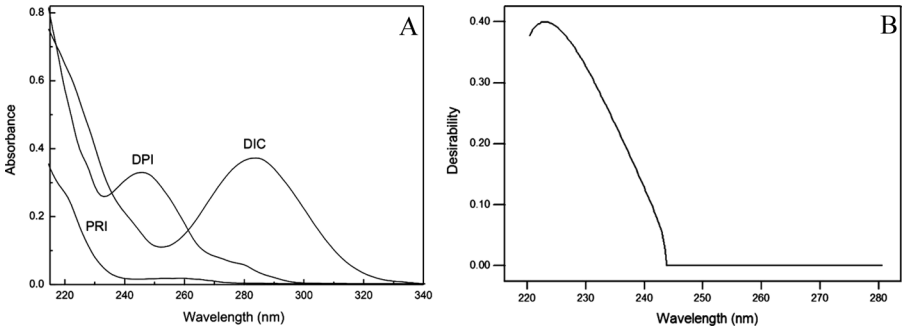

FIGURE 2 (A) UV spectra of pridinol (21 μg mL^{-1}), diclofenac (20 μg mL^{-1}) and DPI (24 μg L^{-1}), dissolved in mobile phase. (B) Desirability plot for the optimization of the detection wavelength.

Considering the high DIC:PRI relationship (12.5:1) and the need to detect minute amounts of DPI, minimization of the response of DIC and simultaneous maximization of the absorbances of PRI and DPI were set as goals. These yielded a global desirability maximum ($D = 0.47$) at 225 nm, which was selected for detection (Figure 2B).

Composition of the Mobile Phase

According to published papers and preliminary experiments in our laboratory, a C_{18} column was chosen for the separation. The optimum composition of the mobile phase was determined at a flow rate of 1.0 mL min^{-1} with nine samples conforming to a 3^2 factorial design. Taking into account that the three analytes have widely different acid-base properties, studied factors were the pH of the sodium phosphate-based aqueous phase, considered at the levels 3.0, 4.5, and 6.0 and the amount of the organic modifier (MeOH:2-propanol, 85:15, v/v), analyzed at 55, 62, and 70%. Addition of 2-propanol demonstrated better peak shapes for PRI.[21] Resolution between adjacent peaks, tailing factors, and the retention times of the first and last eluting compounds were simultaneously evaluated, as system responses.

The resulting response surfaces (Figure 3) revealed the dependence of the retention times of the analytes with the composition of the mobile phase. It was observed that at the lower pH values PRI eluted, followed by DPI and DIC; however, increasing the pH caused simultaneous increase of t_r of DPI and decrease of t_r of DIC, even changing their order of elution. This effect became more important when the proportion of the organic solvent mixture was increased; this delayed PRI, causing DIC to be the first to elute.

The optimum composition for the mobile phase was located employing a global desirability analysis;[25,26] this was found to be a 48:9:43 (v/v/v)

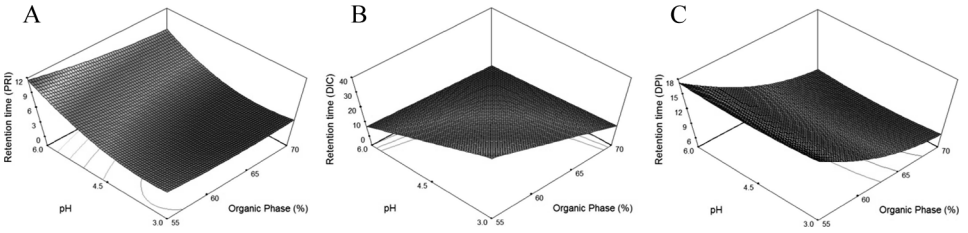

FIGURE 3 Variation of the retention times of (A) PRI, (B) DIC and (C) DPI with the composition of the mobile phase.

mixture of MeOH, 2-propanol and sodium phosphate buffer pH 5.5 (Figure 4A). When tailing factor data were not included in the multivariate calculation, shorter runs were suggested as acceptable solutions, at the expense of separations displaying highly asymmetric peaks. A typical chromatogram is depicted in Figure 4B. Excipients in the commercial formulations did not interfere with the separation of the analytes in tablets and capsules (Figure 4C).

Method Validation

Suitability of the optimized chromatographic conditions for their intended use was demonstrated in agreement with the ICH Q2 guideline.[27] Method linearity and precision were determined with a set of samples conforming a central composite design (Table 1). In the three-factor case, this design contains an embedded two-level full factorial array

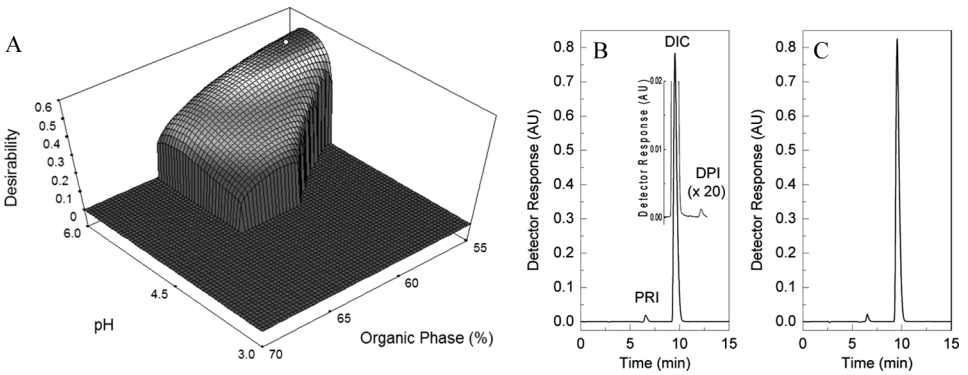

FIGURE 4 (A) Desirability plot depicting the influence of the composition of the mobile phase on the separation of PRI, DIC, and DPI; the white square shows the maximum of the response surface. Chromatographic separations of (B) PRI, DIC, and DPI and (C) a typical chromatogram of a commercial tablet formulation under the optimized conditions.

TABLE 1 Composition of the Analyte Mixtures Employed for Validation Purposes

Sample No.	Analyte Levels (Coded values)			Analyte Concentrations		
				PRI ($\mu g\,mL^{-1}$)	DIC ($\mu g\,mL^{-1}$)	DPI (% DIC)
1	−1.00	−1.00	−1.00	19.7	240	0.082
2	−1.00	−1.00	1.00	19.7	240	0.118
3	1.00	−1.00	1.00	19.7	360	0.118
4	−1.00	1.00	1.00	28.3	240	0.118
5	−1.00	1.00	−1.00	28.3	240	0.082
6	1.00	−1.00	−1.00	19.7	360	0.082
7	1.00	1.00	−1.00	28.3	240	0.082
8	1.00	1.00	1.00	28.3	360	0.118
9	0.00	1.68	0.00	31.2	300	0.100
10	0.00	0.00	1.68	24.0	300	0.130
11	1.68	0.00	0.00	24.0	390	0.100
12	0.00	−1.68	0.00	16.8	300	0.100
13	0.00	0.00	−1.68	24.0	300	0.070
14	−1.68	0.00	0.00	24.0	200	0.100
15–20	0.00	0.00	0.00	24.0	300	0.100

(samples 1–8) and a star (samples 9–14), the points of which are located at a distance $\alpha = 1.68$ from the central point (sample 15). The design allows the evaluation of each factor at five levels with only 15 experiments. Five additional replicates of the central point were employed in order to obtain method repeatability information. Table 2 summarizes method validation results. System suitability parameters and sample stability were also determined.

Specificity

Employing a diode array detector, it was observed that the excipients did not interfere with the quantification of the active principles and DPI. Moreover, the peak purity function, used to further assess the absence of underlying peaks, yielded values exceeding 0.9995 (Table 2), which evidenced that the method is specific for the proposed separation.

Range and Linearity

The regression lines obtained by plotting the peak-areas of the analytes as a function of their corresponding sample concentrations exhibited correlation coefficients exceeding 0.99, and a random distribution of the residuals. Student *t*-test comparisons of the intercepts with zero at a 5% α-error level revealed that they were not significantly different from zero ($t_{\text{Intercept}} = |\text{Intercept}|/\text{SD}_{\text{Intercept}} < t_{18,\ 0.05}$), further confirming method linearity.

TABLE 2 Summary of Method Validation Results

Parameter	PRI	DIC	DPI
Specificity (Peak purity)	0.9997	0.9998	0.9997
Range ($\mu g\,mL^{-1}$)	16.0–32.0	200–400	0.20–0.40[a]
Linearity			
Slope ± SD ($\times 10^8$)	1.46 ± 0.03	6.04 ± 0.08	3.74 ± 0.07
Intercept ± SD ($\times 10^6$)	0.6 ± 0.7	2.1 ± 2.4	0.001 ± 0.001
Correlation coefficient (n = 20)	0.9948	0.9976	0.9964
Accuracy (Bias, %)			
Low, Medium, High concentration[b]	−2.1, +0.5, −1.8	−0.4, −1.4, −0.9	−2.1, −1.4, −0.1
Precision			
Repeatability (RSD, %)[c]	1.9	1.3	3.5
Intermediate precision (RSD, %)[b,d]	1.8	1.0	2.4
Two-way ANOVA		No significant differences (between days and between analysts)	
LOD ($\mu g\,mL^{-1}$)			2.0×10^{-2}
LOQ ($\mu g\,mL^{-1}$)			6.0×10^{-2}[e]

[a]Equivalent to 0.07–0.13% DIC at the central point of the design.

[b]Corresponding analyte levels were PRI: 19.7, 24.0, and 28.3 $\mu g\,mL^{-1}$; DIC: 240, 300, and 360 $\mu g\,mL^{-1}$; and DPI: 0.24, 0.30, and 0.36 $\mu g\,mL^{-1}$.

[c]Six injections of independently prepared samples. Analyte levels were PRI: 24.0 $\mu g\,mL^{-1}$; DIC: 300 $\mu g\,mL^{-1}$; and DPI: 0.30 $\mu g\,mL^{-1}$ (equivalent to 0.10% DIC in the central point of the design, 300 $\mu g\,mL^{-1}$).

[d]A set of nine samples was injected on two successive days by two different analysts.

[e]Equivalent to 0.02% of DIC at the central point of the design.

Accuracy

The simultaneous determination of the analytes in solutions containing low, medium, and high levels of the analytes, prepared by the addition of known amounts of standards to a pre-analyzed tablet matrix, yielded essentially quantitative analyte recoveries (low bias results). This confirmed that the method enables the accurate determination of the analytes.

Precision

The precision of the method was evaluated at the repeatability and intermediate precision levels. In agreement with the ICH Q2 guideline, which suggests the evaluation of six replicate determinations of the analytes at their 100% level,[27] repeatability was determined from data dispersion of the injection of six independent samples (15–20, Table 1) corresponding to the central point of the central composite design. The results (RSD = 1.9% for PRI, 1.3% for DIC, and 3.5% for DPI) were considered acceptable, taking into account that usually accepted RSD values are $\leq$2%, for active principles and $\leq$10% for impurities.

The intermediate precision was assessed by evaluation of nine independent samples containing analyte mixtures at three concentration levels.

This was achieved employing samples corresponding to the full factorial array embedded in the validation central composite design and that of the central point. Injections were performed on two successive days by two independent analysts and the results were analyzed by means of a two-way ANOVA.

No significant differences between days ($F = 3.100$, 0.378, and 0.386) and between analysts ($F = 0.082$, 0.055, and 0.204) were found for PRI, DIC, and DPI, respectively [$F_{(0.95,\ 1,34)} = 4.121$]. Furthermore, essentially quantitative analyte recoveries were obtained, being their observed inter-day RSD values 1.8%, 1.0%, and 2.4% for PRI, DIC, and DPI, respectively (Table 2). All of this confirmed that the method is precise.

LOD and LOQ of DPI

LOD ($2.0 \times 10^{-2}\,\mu g\,mL^{-1}$) and LOQ ($6.0 \times 10^{-2}\,\mu g\,mL^{-1}$) values for the impurity were estimated from the parameters of the corresponding calibration curve (Table 2).[24] The LOQ, which is equivalent to 0.02% of the concentration of DIC found in the central point of the design, was further assessed by injection of samples at this impurity level.

Robustness

Robustness was assessed by performing small changes to the optimized conditions and examining their effect on analytes' recoveries. The method demonstrated to be robust (Figure 5) when the pH was modified between 5.4 and 5.6, the proportion of the organic phase was changed between 55 and 59% and the flow rate was varied between 0.95 and $1.05\,mL\,min^{-1}$.

System Suitability Test

The test, useful for verifying that the system is adequate for the analysis to be performed, was carried out in agreement with official indications.[10,14] Injection of five replicates of a mixed standard solution containing $24\,\mu g\,mL^{-1}$ PRI, $300\,\mu g\,mL^{-1}$ DIC and $0.81\,\mu g\,mL^{-1}$ DPI yielded RSD values of 0.5, 1.2 and 1.0%, respectively, fully complying with the usually accepted values ($\leq 2\%$). The capacity (k'), separation (α) and tailing factors (T_f), as well as column efficiency and resolution (R_s), were within acceptable limits (Table 3).

Solution and Mobile Phase Stability

No significant changes were observed in the content of the analytes during solution stability and mobile phase stability studies conducted after 48 h.[24]

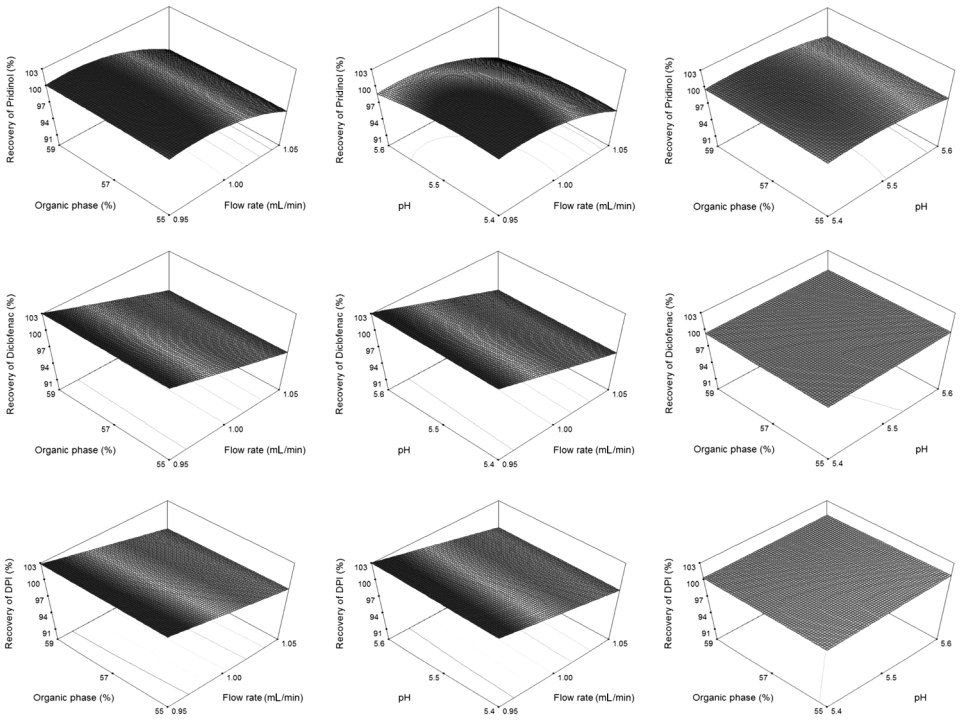

FIGURE 5 Robustness of the proposed method against variations in the flow rate, pH, and percentage of organics in the mobile phase.

Applications

Five commercial brands of tablets and one of capsules were assayed. The observed concentrations of DIC and PRI were within the range of 90–110% of their corresponding labeled contents (Table 4), complying with usually accepted specifications.[10,28] No sample contained DPI levels above the USP official limit for the impurity in DIC tablets (0.1%).[10]

In addition, stressed samples of tablets and granulates containing DIC and the corresponding excipients were also analyzed. While samples

TABLE 3 Results of the HPLC System Suitability Test

Analyte	k'	α	Resolution (R_s)	Tailing Factor (T_f)	Column Efficiency ($N\,m^{-1}$)	RSD (%) for 5 Replicate Injections
PRI	1.74	1.94	8.0 ± 0.1	1.6 ± 0.003	12320 ± 280	0.5
DIC	3.36	1.48	5.6 ± 0.1	1.9 ± 0.008	12550 ± 160	1.2
DPI	4.98			1.3 ± 0.025	32360 ± 640	1.0

TABLE 4 Quantification of the Contents of DIC, PRI, and DPI in Commercial Tablets and Capsules

Samples	Analytes		
	DIC (% ± RSD)[b]	PRI (% ± RSD)[b]	DPI (% ± RSD)[f]
Brand 1[a]	107.1 ± 0.5	91.2 ± 0.4	<LOQ
Brand 2	107.5 ± 1.4	95.7 ± 1.5	<LOQ
Brand 3	104.4 ± 1.8	93.8 ± 1.7	<LOQ
Brand 4	98.2 ± 0.8	95.6 ± 1.4	0.023 ± 0.006
Brand 5	106.9 ± 0.2	98.4 ± 0.5	<LOQ
Brand 4[c]	98.1 ± 0.8	95.2 ± 1.4	0.20 ± 0.02
Brand 4[d]	97.6 ± 0.6	95.8 ± 1.2	0.16 ± 0.03
Mixture 1[c,e]	95.9 ± 1.5	–	0.013 ± 0.006
Mixture 2[d,e]	97.5 ± 0.8	–	<LOQ

[a]Brands 1–4: tablets; Brand 5: soft gelatin capsules.

[b]Data are expressed as percentages with regards to their corresponding label claim (50 mg DIC and 4 mg PRI). RSD values correspond to three replicates (n = 3). 100% of label claim is equivalent to 24.0 μg mL^{-1}PRI and 300 μg mL^{-1} DIC.

[c]Powdered tablets subjected to irradiation with a Philips HPA 400 lamp.

[d]Powdered tablets subjected to irradiation with a 200 W incandescent lamp.

[e]Powdered granulate containing DIC and excipients.

[f]Data are given as % with regards to DIC.

submitted to dry heat and high humidity conditions did not exhibit quantifiable amounts of DPI, the samples of tablets and the granulate subjected to irradiation, showed up to 0.2% DPI. In all cases, no additional peaks which could interfere with the determination of the analytes were observed.

CONCLUSIONS

The results obtained in this work confirm that this experimentally designed HPLC method, properly optimized and validated, fulfills all the pre-established requirements of recognized regulations and is suitable for the quality control of pharmaceuticals containing the DIC-PRI association, being also useful for limiting DPI (diclofenac related compound A) as an impurity in these formulations.

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